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Intra-host Diversity and Evolution of Hepatitis C Virus Endemic to Côte D'Ivoire

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Abstract

Hepatitis C virus (HCV) infection presents an important, but underappreciated public health problem in Africa. In Côte d'Ivoire, very little is known about the molecular dynamics of HCV infection. Plasma samples (n = 608) from pregnant women collected in 1995 from Côte d'Ivoire were analyzed in this study. Only 18 specimens (~3%) were found to be HCV PCR-positive. Phylogenetic analysis of the HCV NS5b sequences showed that the HCV variants belong to genotype 1 (HCV1) (n = 12, 67%) and genotype 2 (HCV2) (n = 6, 33%), with a maximum genetic diversity among HCV variants in each genotype being 20.7% and 24.0%, respectively. Although all HCV2 variants were genetically distant from each other, six HCV1 variants formed two tight sub-clusters belonging to HCV1a and HCV1b. Analysis of molecular variance (AMOVA) showed that the genetic structure of HCV isolates from West Africa with Côte d'Ivoire included were significantly different from Central African strains ($P = 0.0001$). Examination of intra-host viral populations using next-generation sequencing of the HCV HVR1 showed a significant variation in intra-host genetic diversity among infected individuals, with some strains composed of sub-populations as distant from each other as viral populations from different hosts. Collectively, the results indicate a complex HCV evolution in Côte d'Ivoire, similar to the rest of West Africa, and suggest a unique HCV epidemic history in the country.

Keywords

hepatitis C virus; molecular epidemiology; Côte d'Ivoire; West Africa

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped hepatotropic virus that belongs to the genus *Hepacivirus* within the family *Flaviviridae*. The HCV genome is a positive-strand RNA of

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~9,600 nucleotides (nt) consisting of a single open reading frame and two untranslated regions [Choo et al., 1991; Lindenbach and Rice, 2005]. Currently, six HCV genotypes have been recognized and a seventh recently proposed [Simmonds et al., 2005; Nakano et al., 2012]. Owing to the inherent infidelity of the viral RNA-dependent RNA polymerase, the HCV genome accumulates rapidly mutations during replication, leading to a continuous diversification of the intra-host viral population. As a result, each infected individual contains a diverse population of HCV variants or quasispecies [Duffy et al., 2008; Farci, 2011]. Globally, >185 million people are estimated to be infected with HCV, and ~350,000 people die every year from hepatitis C-related liver diseases [EASL, 1999; Mohd Hanafiah et al., 2013]. The severity of the disease varies from asymptomatic infection to cirrhosis and hepatocellular carcinoma (HCC), leading to death [Itskowitz, 2007]. No vaccine to prevent HCV infection is currently available and therapeutic options remain limited, especially in the resource-limited parts of the world [Torresi et al., 2011]. Africa is particularly endemic for HCV infections, with hepatitis C being a pressing public health problem for the entire continent [Madhava et al., 2002; Laurent et al., 2007].

HCV genotypes 1 and 2 (HCV1 and HCV2) are dominant in West Africa, with genotype 1 being prevalent in Nigeria, and genotype 2 circulating broadly across the sub-region [Forbi et al., 2012]. Phylogenetic analysis showed the presence of numerous distinct HCV variants, suggesting that HCV originated in this part of the world. Both HCV genotypes were found to be expanding in West Africa for over 200–350 years. However, the expansion stabilized about the mid-20th century [Forbi et al., 2012]. An investigation of the extent of spread and viral diversity of HCV in West Africa can advance the understanding of the epidemic dynamics of HCV infections not just in Africa but also worldwide as both HCV genotypes 1 and 2 circulate across the globe.

Côte d'Ivoire is geographically located between Ghana and Liberia in West Africa [Zhingoora-Books, 2012]. It has a population of ~22 million people. Information on the spread of HCV in this country has been limited. The HCV antibody seroprevalence was estimated in 1995–1996 to be 3.3% in Abidjan, Côte d'Ivoire [Combe et al., 2001]. A more recent study conducted using serological and molecular assays reported a lower HCV antibody seroprevalence of 1% among pregnant women in Abidjan [Rouet et al., 2004]. Here, we investigated the HCV genetic diversity and evolution in Côte d'Ivoire using conventional and next-generation sequencing technologies.

METHODS

Plasma Samples and Ethical Statement

A total of 608 plasma samples obtained in 1995 from pregnant women in various hospitals during HIV surveillance in Côte d'Ivoire were used for this study. Côte d'Ivoire is located in West Africa, bordering the North Atlantic Ocean, between Ghana and Liberia [Zhingoora-Books, 2012]. The samples for this study were delinked from personal identifiers. The specimens were collected under a CDC IRB-approved protocol and a non-research determination was obtained at the CDC to test anonymized, unlinked samples for HCV.

Nucleic Acid Extraction and cDNA Synthesis

Total nucleic acid was extracted from all plasma samples using the automated Roche MagNA Pure LC robot and the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics, Indianapolis, IN), and eluted. cDNA was generated using the high-temperature capability SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Life Technologies, Carisbad, CA) on an ABI PRISM® 9700 PCR system. The reverse transcription PCR conditions were as follows: 25°C for 10 min, 42°C for 90 min, and 85°C at 5 min.

PCR Amplification and Sanger Sequencing of the Non-Structural Region 5b (NS5b) and Consensus Hypervariable Region 1 (HVR1)

Amplification of the NS5b-gene region was done as we previously described [Forbi et al., 2012] using the LightCycler® 480 software (Version 1.5.0.SP3, Roche, Indianapolis, IN). Samples that were NS5b-positive were subjected to HVR1 amplification. The E1/E2 junction region (306 nt), which contains the HVR1 region, was amplified using the nested PCR protocol described in Ramachandran et al. [2008]. Nested NS5b and HVR1 amplicons derived from the PCR amplification were purified (Millipore multiscreen PCR filter plate, Billerica, MA) and sequenced using their respective nested primers, and the BigDye v3.1 chemistry sequencing kit (Applied Biosystems) by an automated sequencer (ABI 3130xl, Applied Biosystems, Foster City, CA) as previously described [Forbi et al., 2012].

454 Pyrosequencing (GS-Junior) of the HVR1 Region

To generate and detect quasispecies, the HVR1 region of the HCV genome of each sample was amplified independently with fusion primers including the 454-primer key (A and B for forward and reverse primers, respectively), with a different multiple identifier (MID) for each sample, and an HCV-specific sequence using the Roche/454 pyrosequencing technology as we recently described [Forbi et al., 2012]. In this study, instead of the GS FLX platform, we used the 454/Roche GS junior system, with the PCR products purified using size-select gel (Invitrogen), and quantified with the DNA 1000 LabChip on the Agilent 2100 bioanalyzer platform (Agilent Technologies, Inc., Waldbronn, BW Germany or Santa Clara, CA).

To analyze pyrosequencing data, original sequence reads (raw data) were processed using the SFFFILE tools. Sequence reads belonging to each sample were identified and separated using MIDs. Low quality reads were removed. The pyrosequencing data were processed with KEC error correction algorithm, which is highly suitable for rapid recovery of high-quality haplotypes from reads obtained by deep pyrosequencing of amplicons from heterogeneous viruses such as HCV [Skums et al., 2012]. KEC is based on the analysis of a distribution of observed k-mers (sub-strings of reads of a fixed length k). An experimental validation showed that the KEC has a very high-accuracy in finding true haplotypes, estimating frequencies, and correcting false haplotypes [Skums et al., 2012].

Phylogenetic Analysis

Preliminary sequence analysis was conducted using SeqMan and MEGALIGN programs from the Laser-gene DNA and Protein analysis software (version 8.0, DNASTAR, Inc.). The Accelrys GCG Package (Genetic Computer Group, version 11.1-UNIX, Accelrys, Inc.) was

used for further analysis. Nt sequences were aligned using the GCG multiple alignment program PILEUP. HCV genotypes and sub-genotypes were classified based on the NS5b sequence [Smith et al., 1997] by comparing each sequence with published reference sequences from GenBank. These sequences were further compared with other NS5b sequences from other western and central African countries. Maximum likelihood trees were constructed using cleaned HVR1 quasispecies sequences from 454 together with consensus HVR1 sequences generated earlier. Initial phylogenetic trees were built using the Kimura two-parameter nt substation model [Tamura et al., 2004]. Maximum likelihood phylogenetic trees were constructed using MEGA (version 5). Bootstrap values were calculated using 1,000 replicates. The program ARLEQUIN [Excoffier et al., 2005] was used to calculate unbiased estimates of nt diversity according to Nei [1987], and to search for shared haplotypes among patients.

Population Dynamics of HCV in West and Central Africa—Unbiased estimates of nt diversity were calculated according to Nei [1987] using the program ARLEQUIN [Excoffier et al., 2005]. Genetic relationships between West and Central Africa were explored by analysis of molecular variance (AMOVA), as implemented in ARLEQUIN [Excoffier et al., 2005]. We obtained 141 (n = 141) NS5b isolates from Central Africa and 107 from West Africa as previously described [Forbi et al., 2012]. In addition 60 new isolates from Nigeria, West Africa (accession numbers: JQ679028–JQ679087), and 18 from Côte d'Ivoire reported in this study were included in this comparison. The genetic structure was analyzed with consideration of the molecular differences between sequences in addition to differences in their frequencies, resulting in estimates of Φ_{st} (an F_{st} analogue). Significance levels of the genetic variance components were estimated using 10,000 permutations. A *P*-value of 0.05 or less was considered statistically significant.

GenBank numbers—The NS5b and consensus HVR1 sequences reported in this study were submitted to GenBank under accession numbers: KC012572–KC012589 and KC107793–KC107804, KF218817, and KF218818, respectively.

RESULTS

HCV Genetic Diversity

Of the 608 plasma samples collected, 18 were HCV RNA-positive by PCR using the NS5b region (362 nt), giving an overall prevalence of 2.96%. Phylogenetic analysis of the NS5b sequences from the 18 isolates showed that they belonged to HCV1 (n = 12; 66.7%) and HCV2 (n = 6; 33.3%) (Fig. 1). Two HCV1 clusters belonging to HCV1a and HCV1b were also identified (Fig. 1). All NS5b sequences were genetically distinct, with maximum genetic differences among variants of HCV1 and HCV2 being 20.7% and 24.0%, respectively. The identification of such high-genetic diversity indicates either a long evolutionary history of HCV infection or frequent introduction of different HCV lineages. The HCV isolates were inter-mixed with isolates from other West and Central African countries (Fig. 2). Despite their limited number, the Côte d'Ivoire HCV1 variants (IC1, IC12, IC13, IC11, IC14, and IC16) formed two small clusters (Fig. 2). However, all the

HCV2 isolates appeared genetically distant from each other and from variants found in other countries (Fig. 2).

The nt diversity of HCV1 isolates circulating in West and Central Africa was 18% and 15.3%, respectively. For HCV2, the nt diversity was 19.9% and 11.1% for West and Central Africa, respectively. AMOVA revealed that the genetic structure of HCV1 and HCV2 circulating in West Africa as measured by their nt diversity was significantly different from those in Central Africa ($P = 0.0001$).

Intra-Host HCV Diversity

To investigate intra-host HCV heterogeneity, quasispecies analysis of HVR1 was conducted using high-throughput pyrosequencing of 12 HCV1 and 4 HCV2 strains. In average, ~2,063 reads were obtained per individual sample. Phylogenetic analysis of the intra-host HVR1 sequences from all samples revealed the absence of inter-mixing of HCV variants among individuals in the studied population (Fig. 3). Each individual was infected with a population of genetically heterogonous HCV variants (Fig. 3). The extent of intra-host heterogeneity varied broadly. While many samples showed a limited intra-host HCV diversity from ~1.1% to 1.4% as for samples IC7, IC8, and IC12, the maximum genetic distance of 11.7% was observed among the intra-host HVR1 clusters from sample IC11 (Fig. 3), which was similar to distance of 13.0% or 14.3% measured between IC4 and IC5 or IC12 and IC13, respectively. The mean intra-host HVR1 nt diversity was 1.7% ($\sigma = 0.9$) for HCV1 and 2.6% ($\sigma = 1.7$) for HCV2. Wilcoxon rank sum test for equality of means showed that the intra-host diversity of each genotype was similar ($P = 0.4755$). Consensus HVR1 sequences identified by Sanger sequencing of the HVR1-PCR fragments did not match completely any of the corresponding intra-host HVR1 variants in all tested samples (Fig. 3).

DISCUSSION

HCV infections represent a serious and urgent public health problem in Africa where the prevalence is high, the cost of treatment is prohibitive, the reuse of improperly sterilized needles, transfusion of unscreened blood are common and resources to implement public health measures against its spread are limited [Madhava et al., 2002; Prati, 2006; Okwen et al., 2011; Averhoff et al., 2012; Harnois, 2012]. In this study, a prevalence of ~3% of HCV infection (based on PCR detection of HCV RNA) was recorded among samples collected from pregnant women in 1995 from Côte d'Ivoire. An independent study conducted in the same locality at about the same time showed an HCV antibody prevalence of 3.3% in women of childbearing age [Combe et al., 2001]. However, the enzyme immunoassay used in the study has been associated with false-positive results [Njouom et al., 2003; Raghuraman et al., 2003]. A more recent report using molecular assays showed the prevalence of HCV infection in Côte d'Ivoire to be 1% [Rouet et al., 2004], which is three times lower than the rate reported here. The discrepancy can be explained by difference in assay sensitivity, with the assay used here having a detection limit of 50 IU/ml. Nevertheless, the HCV prevalence reported here is lower than currently reported for most West African countries [Segbena et al., 2005; Nkrumah et al., 2011; Forbi et al., 2012].

The number of HCV1 strains found in this study is twice that of HCV2 strains, which seems unusual because HCV2 is more predominant than HCV1 in the other West African countries located westward of Nigeria [Candotti et al., 2003; Zeba et al., 2012]. Although the small number of HCV strains detected here may not accurately represent the actual prevalence of these two genotypes in Côte d'Ivoire, our finding is consistent with the previous report on HCV genotypes among a group of 1,002 pregnant women [Rouet et al., 2004] and blood donors/patients [Bengue et al., 2008] in Abidjan, thus indicating a greater prevalence of HCV1 than HCV2. If confirmed with further investigation, the reverse proportion of HCV1/HCV2 as compared to the neighboring countries suggests a unique epidemic history of HCV in Côte d'Ivoire. As far as we know, this study presents the first publicly available HCV sequences from Côte d'Ivoire and the first report on deep genetic analysis of HCV strains circulating in that country.

HCV isolates identified in this study are highly divergent, indicating their endemicity, and a long natural history in Côte d'Ivoire. HCV2 variants are genetically distant from each other and inter-mixed with variants from other West African countries (Fig. 2), suggesting that Côte d'Ivoire shares the same evolutionary history of HCV2 as the rest of West Africa. However, the presence of two clusters of highly related HCV1 variants belonging to HCV1a and HCV1b indicates that the number of HCV1 lineages circulating in the country is more limited than of HCV2 (Figs. 1 and 2), implying that HCV1 variants were introduced to rather than having originated from Côte d'Ivoire. To result in the reverse proportion of HCV1/HCV2 in this country, this introduction, with predominance of HCV1a and HCV1b strains, either was very extensive or occurred in the background of the low HCV prevalence. The observed grouping of HCV1 variants is reminiscent of the tight HCV1 clusters from Central Africa (Fig. 2), possibly formed as a result of the rapid population expansion of several HCV lineages in the first half of 20th century [Njouom et al., 2007; Pouillot et al., 2008; Markov et al., 2009; Forbi et al., 2010]. These observations are consistent with the hypothesis that the geographic origin of HCV1 is Nigeria [Forbi et al., 2012] rather than Côte d'Ivoire or Central Africa.

Phylogenetic relationships among NS5b and HVR1 sequences from different HCV strains are similar as shown in Figures 1 and 3. For example, pairs of strains, IC4 and IC5, IC9 and IC17, IC12 and IC13, or IC11 and C14, are genetically close in both trees, indicating the accurate estimation of phylogenetic relationships using these two genomic regions and suggesting limited contribution, if any, of recombination to HCV evolution. Intra-host HCV populations, assessed using HVR1 sequences (Fig. 3) were, in general, composed of highly related variants and were not shared among strains. The intra-host HCV1 and HCV2 populations had a comparable degree of genetic diversity. Some intra-host HCV populations were very homogeneous, indicating a short duration of infections in the individuals; for example, IC7, IC8, or IC12, since the degree of HVR1 heterogeneity has been shown to increase significantly after 1–3 years of infection [Ramachandran et al., 2011]. However, several HCV strains contained a variant, which was genetically distant from the major intra-host cluster; for example, IC2 and IC5, indicating that the individuals were infected with a complex population of HCV variants. Two strains, IC11 and IC17, contained three distinct sub-populations, which were as distant from each other (maximal genetic difference of 11.7% and 10.8%, respectively) as viral populations from IC4 and IC5 (13.0% difference) or

IC12 and IC13 (14.3% difference). None of the HVR1 sequences obtained by the direct Sanger sequencing of PCR fragments matched any variants obtained by the next-generation sequencing, indicating that the HVR1 consensus sequences do not represent accurately intra-host viral populations and should be used cautiously for estimation of phylogenetic relationships among HCV strains.

In conclusion, significant differences in the genetic structure of HCV populations circulating in West and Central Africa were found, indicating distinct HCV genetic histories in these geographic regions. Substantial genetic heterogeneity and inter-mixing among HCV2 strains from Côte d'Ivoire and West Africa supports a common origin of HCV2 in this region of Africa. However, identification of tight HCV1 clusters and the unusually high-prevalence of HCV1 over HCV2 suggest a unique epidemic history of HCV infections in Côte d'Ivoire as compared to that in the neighboring West African countries [Candotti et al., 2003; Zeba et al., 2012]. The continued HCV endemicity coupled with a complex intra-host evolution and broad diversity of HCV strains raise concerns regarding the effectiveness of therapy to treat infected persons and control further spread of HCV.

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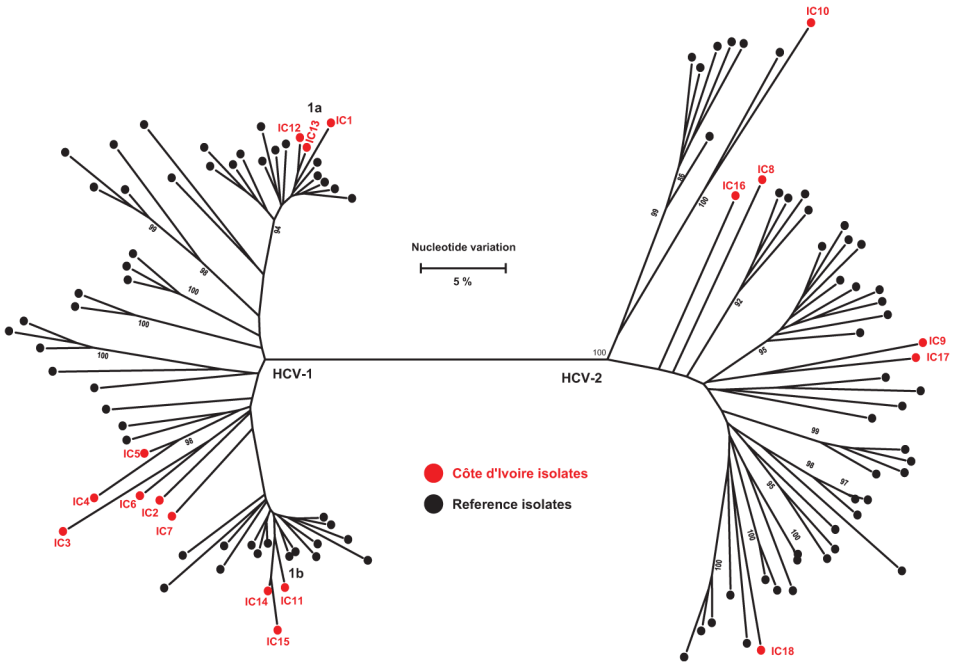

Fig. 1.

Phylogenetic maximum likelihood tree constructed using NS5b sequences. Sequences determined in this study are shown in red. HCV1 and HCV2 are HCV genotypes 1 and 2, respectively. Reference sequences obtained from GenBank are shown in black. Bootstrap values higher than 70% are indicated on major branches. HCV subtype 1a and 1b are also shown.

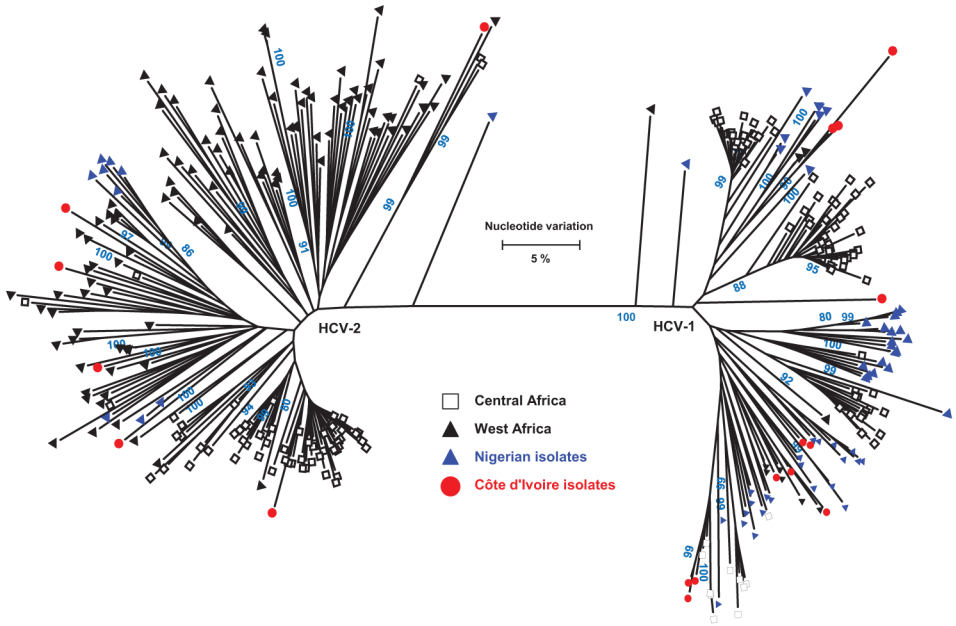

Fig. 2.

Phylogenetic maximum likelihood tree constructed using NS5b sequences from West and Central Africa. Sequences from Central Africa are marked with unfilled squares, and those from West Africa with black triangles. Sequences from Nigeria, West Africa are identified with blue filled triangles. The sequences from this study are marked with red filled circles. Bootstrap values higher than 70% are indicated on major branches.

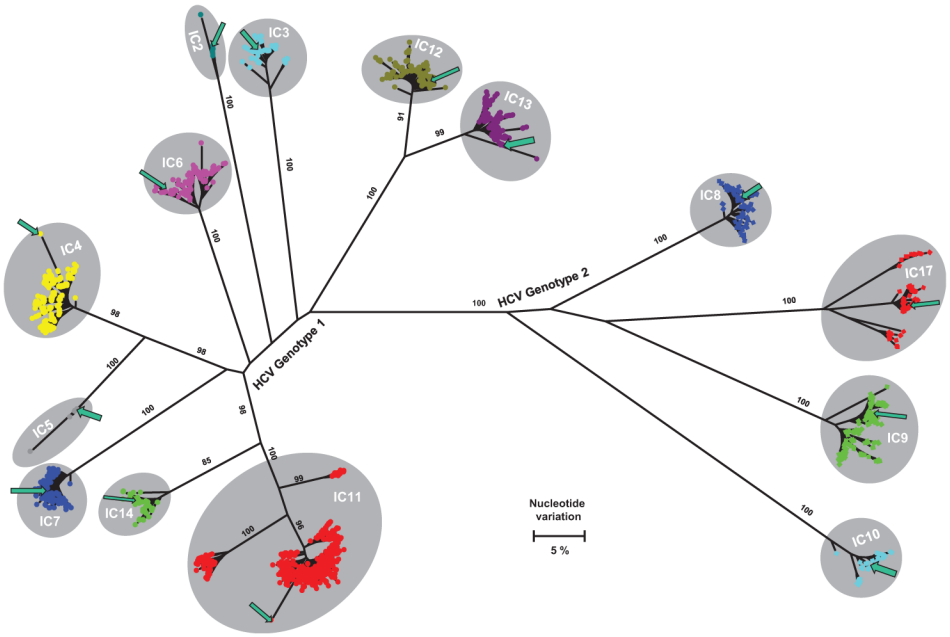

Fig. 3.

Phylogenetic maximum likelihood tree of intra-host HVR1 variants identified in 14 individuals infected with HCV1 and HCV2. All sequences from a single individual are shown using the sample identification code. The arrows indicate consensus HVR1 sequence. Bootstrap values higher than 70% are indicated on major branches.